Phosphorylation of proteins during human myometrial contractions: a phosphoproteomic approach

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Abstract

Phasic myometrial contractility is a key component of human parturition and the contractions are driven by reversible phosphorylation of myosin light chains catalyzed by the calcium (Ca^{2+})-dependent enzyme myosin light chain kinase (MYLK). Other yet unknown phosphorylation or de-phosphorylation events may contribute to myometrial contraction and relaxation. In this study we have performed a global phosphoproteomic analysis of human myometrial tissue using tandem mass tagging to detect changes in the phosphorylation status of individual myometrial proteins during spontaneous and oxytocin-driven phasic contractions. We were able to detect 22 individual phosphopeptides whose relative ratio changed (fold > 2 or < 0.5) in response to spontaneous or oxytocin-stimulated contraction. The most significant changes in phosphorylation were to MYLK on serine 1760, a site associated with reductions in calmodulin binding and subsequent kinase activity. Phosphorylated MYLK (ser1760) increased significantly during spontaneous (9.83 ± 3.27 fold, $P < 0.05$) and oxytocin -induced (18.56 ± 8.18 fold, $P < 0.01$) contractions and we were able to validate these data using immunoblotting. Pathway analysis suggested additional proteins involved in calcium signalling, cGMP-PRKG signalling, adrenergic signalling and oxytocin signalling were also phosphorylated during contractions. This study demonstrates that a global phosphoproteomic analysis of myometrial tissue is a sensitive approach to detect changes in the phosphorylation of proteins during myometrial contractions, and provides a platform for further validation of these changes and for identification of their functional significance.

Key words: Myosin light chain kinase, parturition, phosphoproteomics, myometrium, uterus, contractility

Introduction

Human myometrium at parturition contracts in a phasic manner; regular periods of force are separated by periods of relaxation. This pattern of contractility is important to allow sufficient blood flow through the placenta, providing the fetus with oxygen and removing waste products. Myometrial smooth muscle contractility is driven by action potentials which provoke rapid entry of calcium into the cell through voltage-gated calcium channels. Force is generated after the reversible phosphorylation of myosin light chains (MYL), catalyzed by the Ca^{2+}-calmodulin (Ca-CaM)-dependent enzyme myosin light chain kinase (MYLK) [1]. Phosphorylation of MYL during myometrial contractions has been demonstrated [1, 2], and pharmacological inhibition of MYLK in human myometrium shows that MYLK-induced phosphorylation of MYL is essential for force generation [3]. We have previously demonstrated that the regulatory subunit of myosin phosphatase (MYPT) can be reversibly phosphorylated at an inhibitory site (thr853) during phasic myometrial contractions [2], which contributes to oxytocin-induced Ca^{2+}-sensitisation. Others have described changes in phosphorylation of HSP27, ERK1/2 and h-caldesmon during myometrial contractions [4, 5]. These previous approaches used semi-quantitative mass spectrophotometry (MS) techniques and concentrated on a single or a few phosphoproteins at a time, providing valuable but limited information.

The purpose of this paper was to carry out a global analysis of the myometrial phosphoproteome evoked during spontaneous and oxytocin (OXT)-induced contractions in an attempt to discover novel phosphorylation events involved in regulating contractility. We have used phospho-peptide enrichment, tandem mass tagging (TMT) and mass spectrometry to detect the relative ratio of thousands of phosphopeptides simultaneously. Our approach also allowed us to identify peptides which became phosphorylated after OXT signalling. We

have demonstrated robust contraction-induced phosphorylation of MYLK (ser1760) in myometrial smooth muscle. Pathway analysis revealed additional proteins involved in calcium signalling, cGMP-PRKG signalling, adrenergic signalling and oxytocin signalling were also phosphorylated.

Materials and methods.

Tissue collection.

This study was approved by the North Somerset and South Bristol Research Ethics Committee and all women gave informed written consent. Myometrium was obtained from the upper border of the incision in women undergoing elective (not in labour) sections at term (37-41 weeks gestation). Indications for caesarean sections included fetal malposition, previous section and maternal request. Women with signs of infection were excluded. The tissue was washed in ice-cold isotonic saline and transported to the laboratory where it was used to prepare tissue strips.

Materials.

The following chemicals and reagents were obtained from the indicated sources: Oxytocin was purchased from Merck (Darmstadt, Germany), and butaprost from Cambridge Bioscience (Cambridge, UK). Phospho-MYLK (Ser1760, # 44-1085G) antibody was purchased from Invitrogen, Life Technologies (Paisley, UK). MYLK (# ab76092) antibody was purchased from Abcam (Cambridge, UK), and VASP (# 3112) antibody was purchased New England Biolabs (Hitchin, UK). All other basic chemicals were supplied by Sigma Aldrich (Poole, UK) unless otherwise stated.

Measurement of myometrial contractility.

Contractility measurements were carried out using a four chamber Myobath-II system from World Precision Instruments (WPI, Stevenage, UK) as previously described [2]. For phosphoproteomic studies on relaxed/contracting tissue (Figure 1) spontaneously (SP) contracting myometrial strips were treated with or without 10 nM OXT for a period of approximately 25 minutes. Strips were rapidly frozen either 20 sec after the start of a phasic contraction (SP-CON or OXT-CON), or following a phasic contraction (relaxed; SP-REL or OXT-REL). Tissue was frozen in situ using flat spatulas cooled in liquid nitrogen. The SP-REL strip was used as a control for subsequent phosphoproteomic analysis and immunoblotting quantification.

Tissue homogenisation.

For phosphoproteomics, frozen tissue strips were homogenised in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 8.0, 150 mM sodium chloride, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 1x protease (cOmplete) and phosphatase (PhosTOP) inhibitor mix (Roche Diagnositics Ltd, Burgess Hill, UK)) at approximately 100 mg wet weight/ml using a Polytron homogenizer on ice. Lysates were cleared by centrifugation at 16,000 g for 10 minutes at 4 °C. Protein concentrations were determined using the BCA assay kit (Perbio Science UK, Cramlington, UK) and adjusted to 2 mg/ml. 100 µg of protein was used for phosphoproteomic analysis as described below.

For SDS-PAGE, frozen tissue strips were homogenised in SDS buffer (25 mM Tris pH 6.8, 2 % SDS, 10 % glycerol) at approximately 100 mg wet weight/ml using a Polytron homogeniser at 20 °C. Lysates were immediately heated to 95 °C for 5 minutes, and cleared by centrifugation at 16,000 g for 10 minutes at 20 °C. Protein concentrations were

determined as above before 12.5 mM dithiothreitol (DTT) and 0.002 % bromophenol blue were added prior to electrophoresis.

Phosphoproteomics

Tandem mass tag (TMT) labelling and phospho-peptide enrichment

A 100 µg aliquot of each sample (4 conditions per experiment) was digested with trypsin (2.5 µg trypsin per 100 µg protein; 37 °C, overnight), labelled differentially with TMT four-plex reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Loughborough, UK) and the labelled samples pooled. This pool was then subjected to TiO_2-based phosphopeptide enrichment according to the manufacturer's instructions (Thermo Fisher Scientific).

Nano-LC mass spectrometry (MS)

Enriched phosphopeptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) as described elsewhere [6].

Data analysis

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt Human database using the SEQUEST algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included oxidation of methionine (+15.9949) and phosphorylation of serine, threonine and tyrosine (+79.966) as a variable modifications and carbamidomethylation of cysteine (+57.0214) and the addition of the TMT mass tag (+229.163) to peptide N-termini and lysine as fixed modifications. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery

rate (FDR) of 5%. The TMT tags allowed the abundance of all individual peptides in each of the four samples to be reported, and these data were presented as a ratio relative to the SP-REL sample. Data were then filtered to select only peptides with phosphorylation as a modification, and data from four individual experiments were pooled. Statistical analysis was carried out using Kruskal-Wallis test and Dunn's multiple comparison tests for paired comparisons relative to SP-REL.

SDS-PAGE, immunoblotting and quantification of immunobands.

Tissue lysates (50 μg protein) were resolved using 7.5 % Bis-Tris gels and proteins transferred by immunoblotting as previously described [7]. Membranes were probed initially with phospho-specific antibodies and re-probed with the equivalent total antibody after incubating for 3 x 1 hour in stripping buffer (20 mM glycine, 1 % SDS, pH 2). Bands were quantified by densitometry using Quantity One software (Bio-Rad, Hemel Hempstead, UK), and care was taken to avoid saturation of signals. Raw data were normalised to the using an experimental control for each experiment as detailed in individual figure legends, and the level of phosphorylated MYLK was always normalised using the level of equivalent total MYLK. Data were analysed using Friedman tests and Dunn's multiple comparison tests according to figure legends.

Results

Analysis of protein phosphorylation during spontaneous and oxytocin-induced myometrial contractions.

OXT (10 nM) stimulation of fresh myometrial strips resulted in a 212 ± 21.5 % increase in contractility relative to the pre-treatment period (as determined by area under curve, AUC) , while AUC for spontaneously (SP) contracting strips remained constant throughout the

experimental period (102 ± 9.44 % relative to start). SP or OXT strips were rapidly frozen following (relaxed, REL; 3.17 ± 0.35 min post peak) or during (contracting, CON; after 20 sec) a phasic contraction (Figure 1). The mean time taken for contractions to reach their peak was 34.5 ± 2.39 seconds. This provided us with four experimental groups; SP-REL, SP-CON, OXT-REL and OXT-CON. Lysates from frozen tissue samples were used for phospho-proteomic analysis, and over 4000 phosphopeptides were identified in our samples. Table 1 summarizes the identities of 22 phosphopeptides whose relative ratio changed (either fold >2 or fold < 0.5) in response to spontaneous or OXT- stimulated contraction, or in response to OXT exposure (effect of OXT in the relaxed state). There were significant changes in MYLK (ser1760, $P < 0.001$), CAMK2G (ser325, $P < 0.01$), dematin (DMTN, ser226, $P < 0.05$) and serine/arginine repetitive matrix protein 1 (SRRM1, ser696, $P < 0.05$) phosphorylation which all increased during a spontaneous contraction. MYLK (ser1760), CAMK2G (ser325) and SRRM1 (ser696) increased with OXT stimulation as well as contraction, while calcium-transporting ATPase (ATP2B4, ser1136), tensin-1 (TNS1, ser445), myosin phosphatase regulatory subunit 12B (PPP1R12B, ser839 and ser947), synaptopodin-2 (SYNPO2, thr588/ser604), Kininogen-1 (KNG1, ser332) and KN motif and ankyrin repeat domain-containing protein 2 (KANK2, ser356) all increased with OXT exposure but not contraction. MYLK at an alternative site (ser1772/3/6), ELKS/Rab6-interacting/CAST family member 1 (ERC1, ser21), microtubule-associated protein 1B (MAP1B, ser2200/2), CLIP-associating protein 2 (CLASP2, ser455) and synemin (SYMN, ser1044) decreased in phosphorylation during a spontaneous contraction (all $P < 0.05$). The same proteins, in addition to neuroblast differentiation-associated protein (AHNAK, ser3426) and leiomodin-1 (LMOD1, ser555 and ser508/512/516) decreased in phosphorylation with OXT stimulation. The potential associations between phosphorylated proteins were determined by pathway analysis which placed MYLK phosphorylation central to myometrial contractility (Figure 2). The analysis

revealed statistically significant protein-protein interaction enrichment (P = 3.46e-08) suggesting these proteins may interact *in vivo*.

Confirmation of MYLK phosphorylation during phasic contractions.

The tension generated by spontaneous and oxytocin-augmented contractions is shown in Figure 3A. The phospho-proteomic data demonstrate that phosphorylated MYLK (ser1760) increases significantly during spontaneous (9.83 ± 3.27 fold, $P < 0.05$) and OXT-induced (18.56 ± 8.18 fold, $P < 0.01$) contractions, with modest increases in relaxed tissues after OXT exposure which failed to reach significance (2.24 ± 0.60 fold, Figure 3B). The level of pMYLK (ser1760) in these samples was confirmed by western blotting (Figure 3D and E), and the pattern of phosphorylation was closely coupled to the level of tension in the myometrial strips at the point of freezing (Figure 3A). Interestingly phosphorylation of MYLK at serine 1772/3/6 was markedly decreased with spontaneous contractions (0.45 ± 0.18 fold) and similarly after OXT stimulation, in relaxed and contracted tissue (0.40 ± 0.18 and 0.44 ± 0.15 fold respectively, Figure 3C). These data demonstrate that serine 1760 and serines 1772/3/6 on MYLK are phosphorylated at the peak and trough of a phasic contraction respectively and suggest that separate signalling pathways and protein kinases are responsible.

MYLK is not phosphorylated during butaprost-induced relaxation.

We next investigated whether PKA-activating receptors caused phosphorylation of serine 1760 of MYLK in intact myometrium. This is important because MYLK is a proposed target of PKA-dependent myometrial relaxation on the basis that it is phosphorylated by PKA *in vitro* [8], and pMYLK (ser1760) has decreased myosin kinase activity [9, 10]. The EP2-receptor agonist, butaprost, causes cAMP elevation in human myometrial cells [11].

Phosphorylation of MYLK was measured in butaprost-relaxed tissue (10^{-6} M dose) and compared to the SP-REL and SP-CON levels in matched strips (Figure 4A). While contraction was associated with increased pMYLK (ser1760), there was no increase in the butaprost-treated strips (Figure 4B, C and D), despite increased phosphorylation of the PKA substrate VASP at serine 157. Moreover, the level of pMYLK was closely coupled to the level of tension at freezing (Figure 4B).

Discussion

This paper reports a phosphoproteomic approach to study the mechanism of contractility in human myometrium. We have carried out a global phosphorylation analysis of relaxed versus contracted myometrial tissue using advanced quantitative proteomic techniques. This approach has highlighted the importance of MYLK phosphorylation and revealed several proteins possibly associated with MYLK whose phosphorylation also changes during contractions. We have verified the phosphorylation of MYLK by western blotting, demonstrated that serine 1760 phosphorylation is closely coupled to the generation of force, and that it does not increase during butaprost-induced myometrial relaxation.

The phasic nature of myometrial contraction and relaxation is driven through the reversible phosphorylation of MYL, catalyzed by MYLK. The essential need for MYLK-mediated phosphorylation of MYL has been demonstrated in human myometrium [3], although MYL phosphorylation-independent pathways may be complementary in other species [12]. Our study has revealed phasic changes in the phosphorylation of 18 proteins (4 with multiple phosphorylation sites), with the greatest change in phosphorylation on serine 1760 of MYLK. Pathway analysis of these proteins revealed components of the calcium signalling pathway (KEGG 04020), the cGMP-PRKG signalling pathway (KEGG 04022), adrenergic signalling

in cardiomyocytes (KEGG 04261) and the oxytocin signalling pathway (KEGG 04921), and suggested these proteins may interact *in vivo*. These proteins are likely to be functionally relevant and their involvement in uterine contractions requires further investigation. More specifically, LMOD1 and CAMK2G are involved in the maintenance of a contractile phenotype in vascular smooth muscle cells [13, 14] but their role in myometrial smooth muscle has not been investigated. The myosin phosphatase regulatory subunit family of proteins (PPP1R12A, B and C) is well conserved [15] and is involved in calcium sensitization mechanisms in human myometrium [16]. AHNAK binds to and stimulates L-type Ca^{2+}channel activity and can be regulated by PKA-mediated phosphorylation [17], and provides a link between Ca^{2+}channels and the actin cytoskeleton [18]. Phospholamban (PLN) is a negative regulator of calcium ion import into the sarcoplasmic reticulum and its role in regulating the frequency and amplitude of contractions in rat myometrium has been described [19] but there is little information on the function of this protein in human uterine activity. ATP2B4 (or PMCA) is part of the Ca^{2+} extrusion mechanism responsible for lowering $[Ca^{2+}]_i$ and force after stimulation [20]. KNG1 is cleaved to release bradykinin, which is able to stimulate myometrial contractility [21] by stimulating IP_3-dependent Ca^{2+} release and the bradykinin B2 receptor is expressed in the myometrium at term [22]. Interestingly, the phosphorylation of KANK2 (SRC-1-interacting protein, SIP) by epidermal growth factor has recently been described in human myometrial cells [23]. Phosphorylated KANK2 is a potential mediator of human parturition through stimulation of prostaglandin synthesis [23]. The discovery of multiple accessory proteins whose activity is regulated during contractions represents potential new targets for modulating uterine activity at labour.

It is well established that MYLK activity can be regulated by phosphorylation in addition to its regulation by Ca-CaM. Early studies in tracheal smooth muscle established that

phosphorylation of MYLK at serine 1760 (peptide A) in the calmodulin binding domain interferes with CaM binding and increases Kcam, thereby decreasing kinase activity [9, 10]. Phosphorylation of MYLK at serine 1773 (peptide B) can occur irrespective of CaM binding and both these sites can be phosphorylated *in vitro* by cAMP-dependent protein kinase (PKA) [8] and calmodulin-dependent kinase II (CAMK2) [24]. Studies in myometrial smooth muscle have demonstrated CAMK2-dependent decreases in MYLK activity during a phasic contraction, however phosphorylation of MYLK was not measured [1]. Our observation that MYLK phosphorylation (ser1760) increases during a contraction, shown by phosphoproteomics and verified by western blotting, supports these previous findings and suggest that CAMK2-dependent phosphorylation of MYLK may negatively regulate MYLK activity at high $[Ca^{2+}]_i$ [25]. Activation of the prostaglandin receptor EP2 subtype with butaprost results in cAMP elevation in human myometrium [11, 26]. While butaprost treatment resulted in increased PKA activity as determined by the level of VASP phosphorylation, there was no increase in pMYLK (Ser1760) in butaprost-relaxed tissue. This suggests that phosphorylation of serine 1760 is not a mechanism for agonist-induced myometrial relaxation [27, 28]. It is possible that PKA-dependent uterine relaxation is mediated by other pathways, e.g. activation of potassium channels in myometrial cells [27, 29].

In conclusion we have developed a sensitive method for the quantitative analysis of multiple protein phosphorylation events in human myometrial tissue and have confirmed the central role of MYLK phosphorylation during spontaneous and OXT-enhanced contractions. The functional role of additional proteins listed in Table 1 and the protein kinases/phosphatases involved in their regulation requires further investigation. This paper will stimulate the study

of novel protein targets in the control of uterine contractility to prevent preterm labour or to facilitate induction of labour.

Declaration of interest

There is no conflict of interest to declare.

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Figure legends

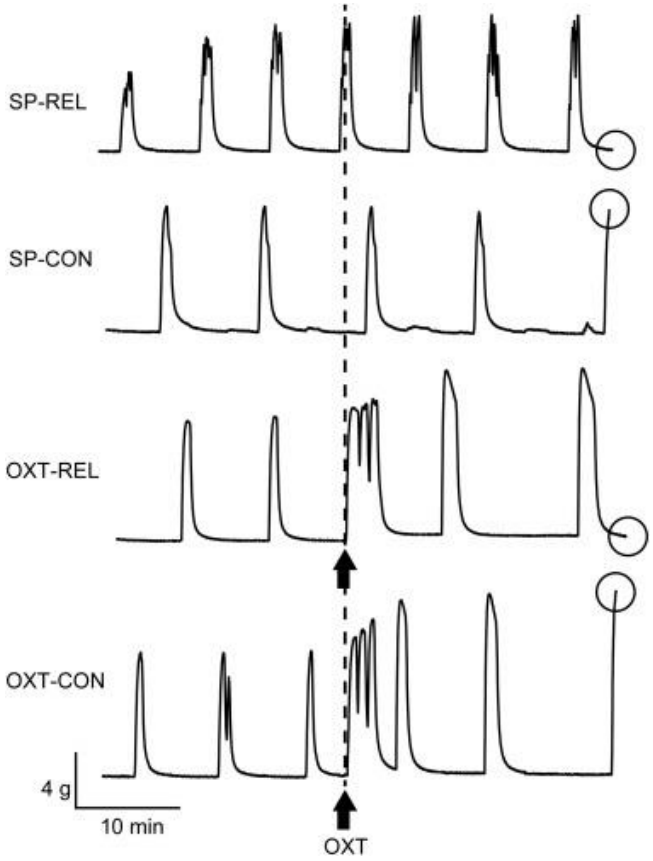

Figure 1. Spontaneous and oxytocin-induced phasic contractions – experimental design for phosphoproteomic study.

Spontaneously contracting myometrial strips (SP) were treated with or without 10 nM oxytocin (OXT). Strips were rapidly frozen after 26.3 ± 2.88 min either 20 sec after the start of a phasic contraction (CON), or immediately following a phasic contraction (REL, 3.17 ± 0.35 min post peak), depicted by open circles. Tissue lysates were subsequently used for phospho-proteomic studies.

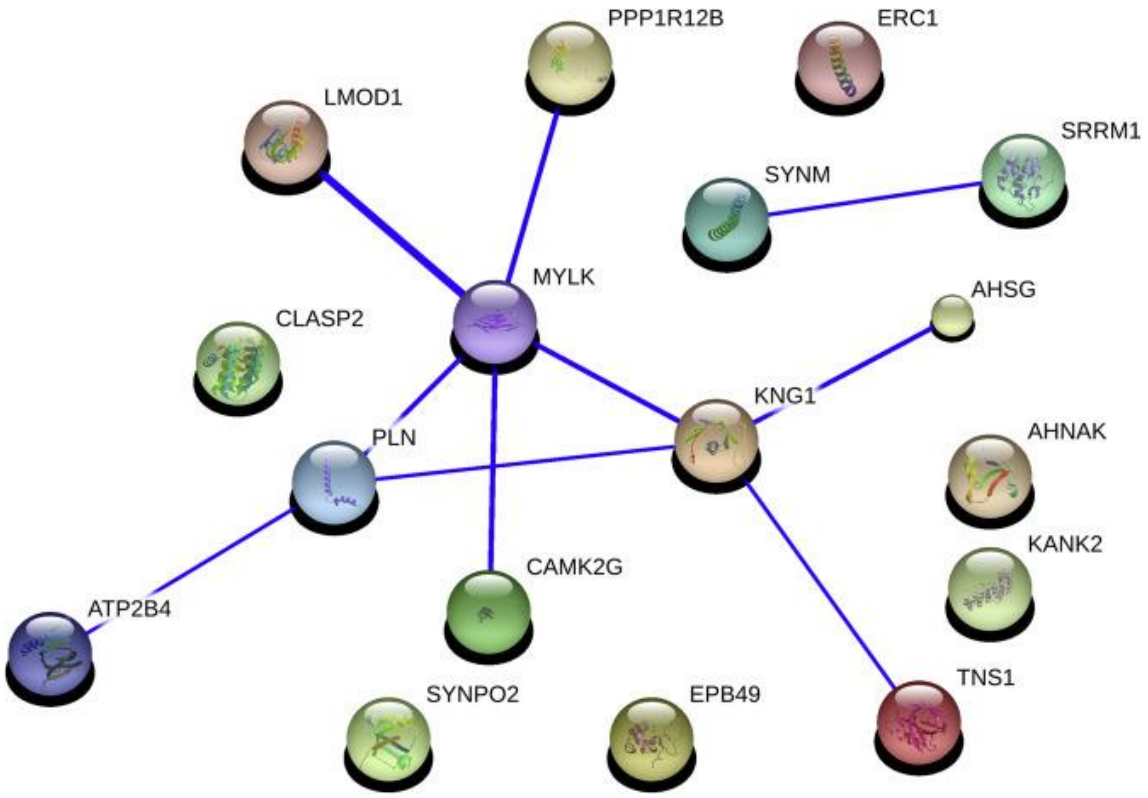

Figure 2. STRING diagram summarising phosphoproteomic data.

The potential associations between proteins phosphorylated during spontaneous and OXT-induced contractions are shown. Stronger associations are represented by thicker lines. This group of proteins show a statistically significant protein-protein interaction enrichment (*P* = 3.46e-08), suggesting these proteins may interact *in vivo*.

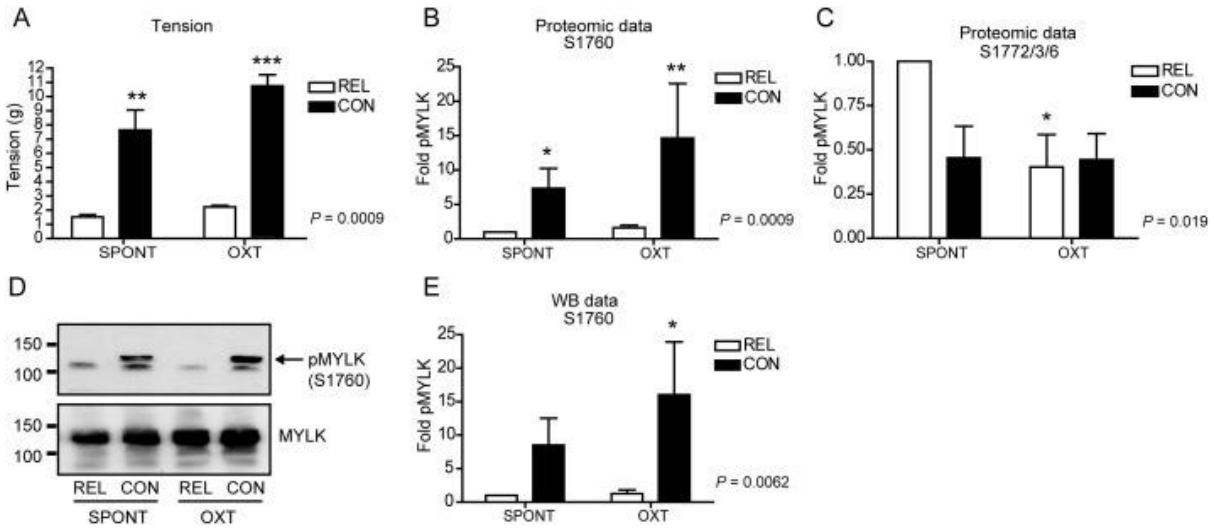

Figure 3. Confirmation of MYLK phosphorylation during phasic contractions by western blotting.

A, myometrial tension (g) at the point of freezing as depicted in Figure 1. Phosphoproteomic data for pMYLK (ser1760) (**B**) and pMYLK (ser1772/3/6) (**C**) expressed as fold change relative to SPONT-REL. Proteins lysates were also resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The levels of pMYLK (ser1760) and MYLK were determined by western blotting (**D**), and quantified by densitometry; pMYLK data were normalised to MYLK levels and expressed as fold change relative to SPONT-REL (**E**). Data were analysed using Friedman tests (*P* value indicated) and Dunn's multiple comparisons tests; *, $P < 0.05$l; **, $P < 0.01$ and ***, $P < 0.001$ for comparison to SPONT-REL, n=4.

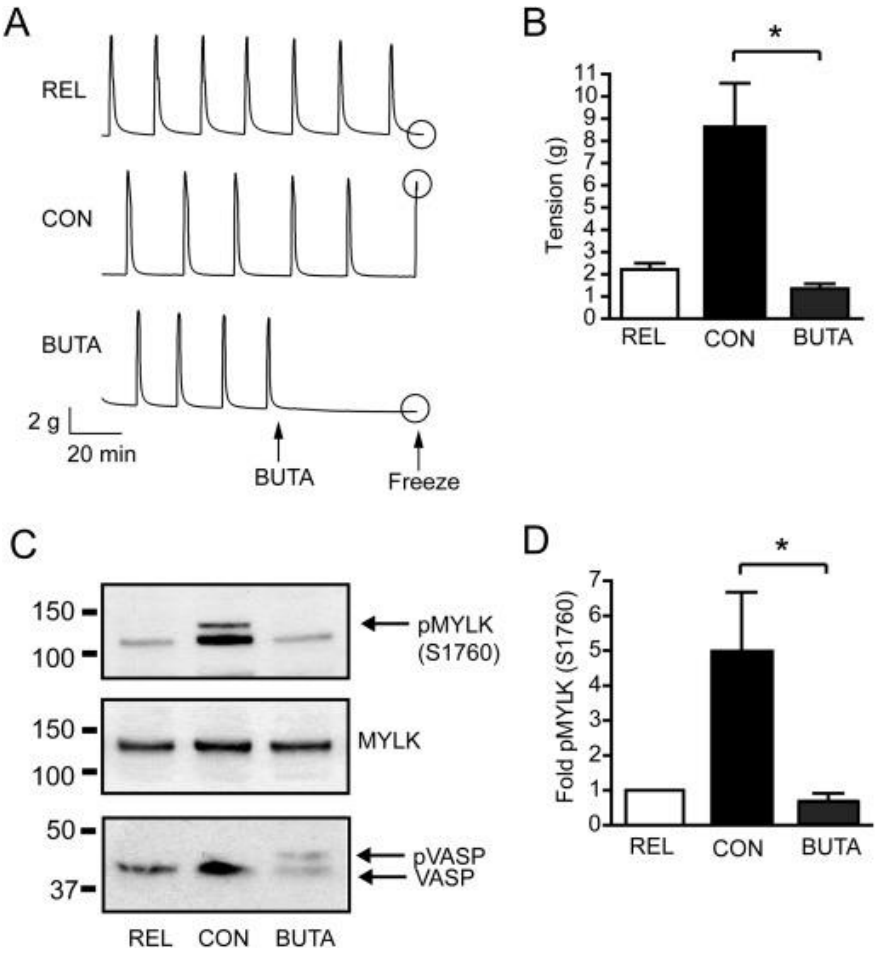

Figure 4. MYLK is not phosphorylated during butaprost-induced relaxation.

A, spontaneously contracting myometrial strips were treated with or without 1 µM butaprost (BUTA) as indicated. Non-treated strips were frozen either 20 sec after the start of a phasic contraction (CON), or immediately following a phasic contraction (REL), and BUTA strips were frozen 20 min after butaprost addition, depicted by open circles. **B**, myometrial tension (g) at the point of freezing. **C**, tissues were homogenized and proteins resolved by SDS-PAGE; phosphorylated MYLK (ser1760), MYLK and VASP were detected by western blotting. Phosphorylated VASP (ser157) is seen as a slower migrating band. **D**, pMYLK (ser1760) levels were quantified by densitometry and data were normalised to the level of MYLK. Statistical significance of tension and phosphorylation data were determined by Friedman tests and Dunn's multiple comparison tests; * $P < 0.05$, n=4.

Table 1.

Phosphoproteomic analysis of relaxed and contracting human myometrial tissue<comma> with and without oxytocin stimulation.

Description	Gene	Accession	Sequence	Site (s)	SP-CON	OXT-REL	OXT-CON	P			
		Mean ratio	SEM	Mean ratio	SEM						
Myosin light chain kinase	MYLK	Q15746	LSsMAMISGLSGR	S1760	9.83*	3.27	2.24	0.6	18.56**	8.18	0.0001c
Calcium/calmodulin-dependent protein kinase II gamma	CAMK2G	Q13555	QSSAPAsPAASAAGLAGQAAK	S325	3.77	1.74	2.63	1.16	3.10*	1.52	0.010 b
Alpha-2-HS-glycoprotein	AHSG	P02765	CDSSPDsAEDVR	S138	2.67	0.96	1.49	0.27	2.25	0.78	0.073
Dematin	DMTN	Q08495	RGAEEEEEEEDDDsGEEMK	S226	2.43	1.16	1.19	0.19	2.67*	0.87	0.034a
Serine/arginine repetitive matrix protein 1	SRRM1	Q8IYB3	APQTSSsPPPVR	S696	2.35	0.68	2.57	0.58	3.10*	0.98	0.017a
KNG1 protein	KNG1	P01042	ETTCSKEsNEELTESCETK	S332	2.32	0.73	1.7	0.2	2.82*	0.66	0.053
Cardiac phospholamban	PLN	P26678	RAsTIEMPQQAR	S16	2.14	0.4	2.13	0.63	2.14	0.18	0.088
ELKS/Rab6-interacting/CAST family member 1	ERC1	Q8IUD2	VEPSSQSPGRsPR	S21	0.48	0.09	0.34	0.1	0.4	0.01	0.047a
Microtubule-associated protein 1B	MAP1B	P15205	HMDPPPAPVQDRsPsPR	S2200<comma> S2202	0.47	0.12	0.33*	0.08	0.38	0.09	0.016a
Myosin light chain kinase	MYLK	Q15746	ssTGsPTSPLNAEK	S1772<comma> S1773<comma> S1776	0.45	0.18	0.40*	0.18	0.44	0.15	0.019a
Synemin	SYNM	O15061	QRsPAPGSPDEEGGAEAPAAGIR	S1044	0.42	0.09	0.40*	0.12	0.46	0.1	0.017a
CLIP-associating protein 2	CLASP2	O75122	VVSQsQPGsR	S455	0.41	0.09	0.42	0.16	0.57	0.08	0.045a
Calcium-transporting ATPase	ATP2B4	P23634	LVPsSSYVAVAPVK	S1136	0.7	0.04	6.94	2.72	9.1	6.24	0.0002c
Tensin-1	TNS1	Q9HBL0	LLsGFGLER	S445	1.23	0.15	6.55	1	7.59	1.67	0.010 b
Myosin phosphatase regulatory subunit 12B	PPP1R12B	O60237	LEsGGSNPTTSDSYGDR	S839	0.94	0.26	5.21	0.91	5.32	1.68	0.001c
Synaptopodin-2	SYNPO2	Q9UMS6	tAKPFPGSVNQPATPFsPTR	T588<comma> S604	1.78	0.36	4.14*	2.05	3.55*	1.02	0.007 b
KN motif and ankyrin repeat domain-containing protein 2	KANK2	Q63ZY3	AQsLEPYGTGLR	S356	1.05	0.06	1.99	0.31	2.12	0.19	0.010 b
Myosin phosphatase regulatory subunit 12B	PPP1R12B	O60237	KMsEMEEEMK	S947	1.09	0.23	1.79	0.25	2.26*	0.21	0.010 b
Neuroblast differentiation-associated protein	AHNAK	Q09666	VSMPDVELNLKsPK	S3426	0.56	0.08	1.29	0.22	0.47*	0.09	0.001c
Calcium/calmodulin-dependent protein kinase II gamma	CAMK2G	Q5SWX3	QEtVECLR	T287	0.53	0.14	0.40*	0.05	0.71	0.18	0.031a
Leiomodin-1	LMOD1	P29536	NSLsPATQR	S555	0.86	0.4	0.35*	0.14	0.36	0.09	0.032a
Leiomodin-1	LMOD1	P29536	GsPKPsPQPsPKPSPK	S508<comma> S512<comma> S516	0.63	0.34	0.20*	0.06	0.21	0.06	0.016a